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Date: March 22, 2002

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371

International Application No.:	PCT/US00/40704
International Filing Date:	August 21, 2000
Priority Date Claimed:	August 19, 1999
Title of Invention:	**PRESERVATION OF BACTERIAL CELLS AT AMBIENT TEMPERATURES**
Applicants for DO/EO/US:	Victor Bronshtein, Charles Isaac, Gordana M. Djordjevic,

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.

2. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).

3. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

4. (X) A copy of the International Application as filed (35 USC 371(c)(2)) not necessary as the application was filed with the RO/US.

5. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3)) have not been made and will not be made.

6. (X) **A FIRST PRELIMINARY AMENDMENT.** At page 1, line 4, prior to the first line of text, please add the following paragraph:

> This application is the U.S. National Phase under 35 U.S.C. § 371 of International Application No. PCT/US00/4074 which claims priority under 35 U.S.C. § 119(e) from U.S. Provisional Application No. 60/149,795.

7. (X) The present application qualifies for small entity status under 37 C.F.R. § 1.27.

8. (X) A return prepaid postcard.

9. (X) A PTO Form 1449 listing five (5) references that were cited in the ISA. As these references are already of record in this application, no copies of these references are submitted. The Form 1449 is submitted solely for the convenience of the PTO.

10. (X) The following fees are submitted:

				FEES
	BASIC FEE			$355
CLAIMS	**NUMBER FILED**	**NUMBER EXTRA**	**RATE**	
Total Claims	12 - 20 =	0 ×	$9	$0
Independent Claims	1 - 3 =	0 ×	$42	$0
Multiple dependent claims(s) (if applicable)			$140	$0
Fee for Petition to Revive				$620
	TOTAL OF ABOVE CALCULATIONS		$975	

11. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.

12. (X) A check in the amount of $975.00 to cover the above fees is enclosed.

13. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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PRESERVATION OF BACTERIAL CELLS AT AMBIENT TEMPERATURES

Background of the Invention

Preservation by drying has been known for thousands of years. Modern refinements of this art are evident in freezing, freeze-drying, drying from liquid, and desiccation. The key step is to remove the water that allows the harmful interactions to occur. The art has been successfully carried out on many biological materials including microorganisms. However this development has been variable in both type of organisms that can be successfully preserved and variability with preservation of that organism.

Conventional preservation methods, including freeze-drying and spray drying, provide only limited success in delivery of metabolically active cells present at high densities. Cell injuries inherently associated with these methods of preservation result in starter cultures with low metabolic activity and cell density, causing long "lag" phases in commercial fermentations and economic losses. Recently, Bronshtein has developed an alternative foam-drying process for the long-term stabilization of sensitive biological materials at ambient temperatures in the dry state (U.S. Patent No. 5,766,520). The foam-drying process addresses many of the drawbacks associated with freeze- or spray-drying and results in much lesser damage to numerous biological materials, including starter cultures.

At the present time, bacteria are utilized in a wide range of commercial applications. Lactic acid bacteria cultures are used to produce cheese, yogurt, and other dairy products. Lactobacillus acidophilus, Bifidobacteria, E. coli and other types of bacteria are extensively used as probiotics. Live attenuated bacteria are extensively used to vaccinate different domestic animals and humans. Genetically altered bacteria are widely used as expression hosts for a variety of proteins and products. Unfortunately, broader applications of bacteria and other cell cultures are limited due to deficiencies in conventional preservation methods that do not allow effective stabilization and therefore distribution of cells at room and higher temperatures.

Cell cultures have become increasingly significant in economic and commercial importance. The expression of recombinant products in cell culture systems is becoming widespread and has greatly increased the number of products available for use in industry and medicine. Practical incorporation of these cultures into commercial products has been hampered by their fragile nature and the special requirements in handling them.

The conventional approach for protection of biological materials during desiccation is based on the water replacement hypothesis, first introduced by Webb ("Bound Water in Biological Integrity". Springfield, Ill., C. C. Thomas, 1965). Crowe, Roser and their collaborators, as well as several other groups, reported that disaccharides (sucrose and trehalose) are effective as protectors against desiccation-induced damage because they effectively replace "water of hydration" at the polar groups of biological molecules. Bronshtein and Leopold (1996, Cryobiology 33(6):626-7) showed that some minimal amount of disaccharides (~10 g per g enzyme) is required to provide macromolecule stabilization at high temperatures (37° and 50° C). These observations are in agreement with the conventional belief that desiccation-induced damage results from an increase in hydration forces between biological molecules in the absence of protective fillers (i.e. sugars) and that cells should be filled with significant amounts of

sugar or other protectors to survive desiccation. Because most disaccharides permeate slowly into cells, the generation of sufficient concentrations of intracellular protectants remains a significant challenge.

Accordingly, there is a need for a method of increasing the tolerance of bacterial cells to desiccation in large, industrial scale volumes. The present invention addresses various aspects of enhancing both the tolerance of bacterial cell cultures to desiccation by a scaleable foam-drying procedure and the survival of dried cells during subsequent storage at ambient temperatures. The disclosed methods are based on the inventors' observations that certain modifications in bacterial cell culture conditions prior to foam-drying enhance the cells subsequent ability to survive desiccation and storage, possibly through the induction of intracellular protectant molecules.

Summary of the Invention

A scaleable method for preserving bacterial cells in a dried state is disclosed. The method comprises culturing the bacterial cells under modified fermentation conditions that inhibit fermentation yield relative to optimal fermentation conditions and also enhance the cells' ability to survive dehydration, and drying the cell suspension by boiling under vacuum to form a mechanically-stable foam.

The modified fermentation conditions comprise fermentation parameters selected from the group consisting of temperature, pH, osmotic pressure, divalent cation concentration, cell density, nutrient concentration, oxygen concentration, and nitrogen concentration.

In one variation, two or more of the fermentation parameters are modified to enhance the cells' ability to survive dehydration.

In another variation, the modified fermentation conditions may be applied for only a portion of the culturing step.

The modified fermentation conditions may comprise at least increasing osmotic pressure of the media to 1.2-10 times isotonic pressure. The osmotic pressure may be increased by adding non-permeable solutes, permeable solutes, metabolized or non-metabolizable solutes. In a preferred variation, the osmotic pressure is increased by adding at least one product of cell metabolism.

The modified fermentation conditions may comprise an increase or a decrease of about 0.5-4.5 pH units pH from an optimal pH.

The modified fermentation conditions may also comprise maintaining cultures to various non-optimal growth phases, such as early or late stationary phase, or early or late logarithmic phase.

The modified fermentation conditions may also comprise a change in temperature within a range of 1-15° C from optimal.

Brief Description of the Drawings

Figure 1 shows stability of L. acidophilus harvested at either log or stationary phase.

Figure 2 shows stability of DSM L. acidophilus mixed with prebiotic and stored at room temperature.

Figure 3 shows a comparison of Bordetella survival following preservation by freeze-drying and by foam-drying.

Others have shown that bacterial cultures harvested in a stationary phase of growth survive desiccation better than cultures desiccated in a logarithmic phase. See for example, Legg U.S. Patent No. 5,728,574. Similarly, the use of nutrient starvation, heat shock and "osmoadaptation" by salt addition resulted in higher survival of Pseudomonas flourescens preserved by desiccation at 20° C. Legg suggested that shock related production of intracellular trehalose is responsible for the increase in cell survival in his experiments. Tunnacliff et al. (WO 98/24882) also indicated that expression of trehalose production genes is a solution for bacteria survival in the dry state. Other modifications in fermentation protocols focus primarily on increasing the osmotic strength of the medium to increase the accumulation of internal osmotic protectants such as trehalose, glutamate and betaine. This accumulation results from the addition to salts from 0.0-0.6 M salts to present an osmotic stress to the cells. Glaasker et al. 1996 Osmotic regulation of intracellular solute pools in Lactobacillus plantarum. J. Bacteriol. 178:575-582; Horlacher et al. 1996 Characterization of a cytoplasmic trehalose of Escherichia coli. J. Bacteriol. 178:6250-6257; Ogahara et al. 1995 Accumulation of glutamate by osmotically stressed Escherichia coli is dependent on pH. J. Bacteriol. 177:5987-5990.

The present invention discloses effective preservation at ambient temperatures of bacterial cell cultures in industrial scale volumes by combining the both modification of conventional fermentation conditions, as suggested above, with the scaleable foam-drying preservation technology taught by Bronshtein, U.S. Pat. No. 5,766,520; incorporated herein in its entirety by reference thereto.

Modification of fermentation was effective in enhancing preservation survival for a number of microorganisms, including: Lactococcus lactis subsp. cremoris ATCC 19257; Lactic acid bacteria, Lactobacillus acidophilus ATCC 4356, catfish vaccine, attenuated Edwardsiella ictaluri; kennel cough in dogs, attenuated Bordetella bronchiseptica; strangles in horses, attenuated Streptococcus equi; salmonellosis, attenuated Salmonella choleraesuis; and others. The common feature of the organisms preserved utilizing fermentation modification and subsequent preservation by foam-drying, is accumulation of "internal protectants", including carbohydrates and peptides that support viability upon drying. The modification of fermentation serves to increase the production and accumulation of these compounds through the means herein described. The assumption of this process is that the organisms have the genetic capability to produce such "internal protectants". The modifications of fermentation described herein are only applicable if the genetic potential for expression of internal protectants is demonstrated. If the organisms do not have the genetic capability for such protectants, the genes responsible can be provided for these organisms. The genetic transformation, or induction of transferred genes, may be accomplished by standard techniques. Preferably, the transferred gene(s) are expressed only in response to the modified fermentation techniques disclosed herein.

The methods and compositions of the present invention may encompass bacterial cells other than those specifically used in the below working examples. Further, other types of cell cultures are also deemed amenable to preservation using the disclosed techniques, such as for example, eubacteria, archaebacteria, protozoa, plankton

(phyto- and zoo-), algae, fungi, mammalian (B-cells, fibroblasts, myoblasts, etc.) and insect cells. The preserved cells may be used for attenuated bacteria or microorganizm-based vaccines, test kits and/or bioassays that require indicator cells, as well as any pre-packaged recombinant expression systems that use a defined cell line.

Foam-Drying Process – In a preferred embodiment of the present invention, preservation is carried out by the foam-drying process detailed in U.S. Patent No. 5,766,520 to Bronshtein. This so-called foam-drying protocol may include adding excipients, including carbohydrates and disaccharides to the biological suspension, and foaming the sample to thin films resulting in preservation by drying at ambient or higher temperatures. The bacterial cells were generally mixed in preservation solutions prior to drying. The suspensions were dried under vacuum and foamed to form thin films. Vitrification (glass formation) may or may not occur depending on the drying and storage conditions as detailed by Bronshtein, U.S. Pat. No. 5,766,520. The dried samples were stored under vacuum.

A variety of polyols and polymers are known in the art and may serve as protectants as long as they enhance the ability of the cells to withstand drying and storage. Indeed, the protectant molecules provide other advantages during preservation (see infra, as an aid to generating mechanically stable foams) besides stabilizing cells during dehydration. More particularly, the protectants in accordance with the present invention may include, without limitation, simple sugars, such as sucrose, glucose, maltose, sucrose, xylulose, ribose, mannose, fructose, raffinose, and trehalose, non-reducing derivatives of monosaccharides and other carbohydrate derivatives, sugar alcohols like sorbitol, synthetic polymers, such as polyethylene glycol, hydroxyethyl starch, polyvinyl pyrrolidone, polyacrylamide, and polyethyleneamine, and sugar copolymers, like Ficoll and Dextran, and combinations thereof. Low molecular weight proteins that are soluble in the cell suspension may also serve as protectants.

In one preferred variation of the present invention, the protective composition may comprise mixtures of a low molecular weight sugar, a disaccharide, oligosaccharide and polymer, including a biological polymer. The low molecular weight sugar is used to penetrate and protect intracellular structures during dehydration. Low molecular weight, permeating sugars may be selected from a variety of ketoses, which are non-reducing at neutral or higher pH, or methylated or ethylated monosaccharides. Among the non-reducing ketoses, are included: the six carbon sugars, fructose, sorbose, and piscose; the five carbon sugars, ribulose and xylulose; the four-carbon sugar, erythulose; and the three-carbon sugar, 1,3 dihydroxydimethylketone. Among the methylated monosaccharides, are the alpha and beta methylated forms of gluco, manno, and galacto pyranoside. Among the methylated five carbon compounds are the alpha and beta forms of arabino and xylo pyranosides. Disaccharides, like sucrose, are known to be effective protectants during desiccation because they replace the water of hydration on the surface of biological membranes and macromolecules. In addition, sucrose and/or other fillers may be effectively transformed into a stable foam composed of thin amorphous films of the concentrated sugar when dried under vacuum.

Combining monosaccharides with disaccharides and oligosaccharides effectively prevents crystallization of the oligosaccharides during dehydration. A polymer may be employed to increase the glass transition temperature (Tg) of the dehydrated mixture, which may be decreased by inclusion of the low molecular weight monosaccharides. Any biological polymers that are highly soluble in concentrated sugar solutions may be employed. For example, polysaccharides, like

Ficoll, and Dextran, and synthetic polymers, like hydroxyethyl starch, polyethylene glycol, polyvinyl pyrrolidone, polyacrylamide, as well as highly soluble natural and synthetic biopolymers (e.g. proteins) will help to stabilize biological membranes and increase Tg.

To facilitate scale-up of the preservation process to accommodate commercially useful volumes, desiccation of the bacterial cell cultures is preferably accomplished by foam-drying to form a mechanically stable porous structure by boiling under a vacuum. The drying step may be carried out at temperatures in the range of about -15 to 70 C. The mechanically stable porous structure, or foam, consists of thin amorphous films of the concentrated fillers. Preservation by foam formation is particularly well suited for efficient drying of large sample volumes, before vitrification, and as an aid in preparing a readily milled dried product suitable for commercial use.

In a variation of the present invention, dilute culture suspensions may be concentrated by partially removing the water to form a viscous specimen before foam-drying under vacuum. This initial concentration step can be accomplished either before or after introduction of the sample into the processing chamber, depending on the concentration method chosen. Alternatively, some samples may be sufficiently viscous after addition of the protectant molecules, and therefore not require any initial concentration. In situations where it is desirable to increase the viscosity of the samples, methods contemplated for use in initial concentration include freeze-drying, evaporation from liquid or partially frozen state, reverse osmosis, other membrane technologies, or any other concentration methods known in the art.

The samples are subjected to vacuum, to cause them to boil during drying at temperatures substantially lower than 100 C. In other words, reduced pressure is applied to solutions or suspensions of biologically active materials to cause the solutions or suspensions to foam during boiling, and during the foaming process further solvent removal causes the ultimate production of a mechanically-stable open-cell or closed-cell porous foam.

While low vacuum pressures (in the range of 0.1-0.9 atm) may be applied to facilitate the initial evaporation to produce a concentrated, viscous cell suspension, much higher vacuum pressures (0-24 Torr) are used to cause boiling. The vacuum for the boiling step is preferably 0-10 Torr, and most preferably less than about 4 Torr. Boiling in this context means nucleation and growth of bubbles containing water vapor, not air or other gases. In fact, in some solutions, it may be advantageous to purge dissolved gases by application of low vacuum (about 0.1-0.9 atm) at room temperature. Such "degassing" may help to prevent the solution from erupting out of the drying vessel. Once the solution is sufficiently concentrated and viscous, high vacuum can be applied to cause controlled boiling or foaming. Concentration of the protectant molecules recited above, in the range of 5-70% by weight, during initial evaporation aids in preventing freezing under subsequent high vacuum and adds to the viscosity, thereby facilitating foaming while limiting uncontrolled eruptions.

Rapid increases in pressure or temperature could cause a foam to collapse. In this case, to enhance the mechanical stability of the porous structures, surfactants may be added as long as those additives do not interfere with the biological activity of the solute intended for conversion to dry form. Moreover, drying of the protectant polymers also contributes to the mechanical stability of the porous structures. Foams prepared according to the present invention may be stored in the processing chamber under vacuum, dry gas, like N_2 atmosphere and/or chemical desiccant, prior to subsequent processing operations, (e.g. stability drying, vitrification or milling).

The following examples illustrate the foam-drying process as applied to bacterial cell cultures that have not been subjected to modification of fermentation conditions prior to drying:

(1) A mixture (100 l) containing 50 l of 50% by weight sucrose and 50 l of an ice nucleating bacteria suspension, (INB) Pseudomonas syringae ATCC 53543, were placed in 1.5 ml plastic tubes and preserved by drying at room temperature. First, the samples were dried for 4 hours under low vacuum (0.2 atm). Second, the samples were boiled during 4 hours under high vacuum (<0.01 atm). After boiling under high vacuum, a mechanically-stable porous structure was formed. Third, the samples were stored during 8 days over DRIERITE under vacuum at room temperature.

After 8 days, the samples were rehydrated with 500 l water. Rehydration of the samples containing the dry foams was an easy process that was completed within several seconds. Then the samples were assayed for ice nucleation activity in comparison with control samples. There was no significant difference between the ice nucleating activity per 1,000 bacteria in the samples preserved by the present method versus the control samples.

(2) A sample containing a 1:1 mixture of a concentrated suspension of ice nucleating bacteria (INB) Pseudomonas syringae ATCC 53543 and sucrose has been used. The sample was mixed until all sucrose crystals were dissolved, so that the final suspension contained 50 wt% sucrose. The suspension was placed in 20 ml vials at 2 g per vial. The vials were dried inside a vacuum chamber. The vials were sitting on the surface of a stainless steel shelf inside the chamber. The shelf temperature was controlled by circulating ethylene glycol/water antifreeze at a controlled temperature inside the shelf. Before the vacuum was applied the shelf temperature was decreased to 5 C. Then, the hydrostatic pressure inside the chamber was decreased to 0.3 Torr. Under these conditions the suspension boiled for 30 min. The temperature of the shelf was then slowly (during 30 min) increased up to 25 C. Visually stable dry foams inside the vials under these experimental conditions were formed within 3 hours. Subsequently, the samples were kept under the vacuum at room temperature for one more day. Ice nucleating activity of preserved INB was measured after the samples were rehydrated with 10 ml of 0.01 M phosphate buffer. Ice nucleating activity was measured as a concentration of ice nucleating centers that can nucleate an ice crystal in a 10 l buffer drop during 5 minutes at -5 C. The results of the assay show ice nucleating activity in the preserved samples was equivalent to that observed in fresh controls.

(3) A concentrated INB suspension was frozen to -76 C for future use. The frozen suspension (6 g) was thawed at 4 C and mixed with 4 g of 9:1 sucrose: maltrin mixture. The sample was mixed until the sugars were completely dissolved, so that the final suspension contained 35 wt% sucrose and 4 wt% maltrin. The suspension was placed inside 20 ml vials at 2 g per vial. The vials were dried inside a vacuum chamber. The vials were sitting on the surface of stainless steel shelf inside the chamber. The shelf temperature was controlled by circulating ethylene glycol/water antifreeze at a controlled temperature inside the shelf. Before the vacuum was applied the shelf temperature was decreased to 5 C. The hydrostatic pressure inside the chamber was then decreased to 0.5 Torr. Under such conditions, the suspension boiled for 30 min. The temperature of the shelf was then slowly (during 30 min) increased up to 25 C. Visually, the formation of stable dry foams inside the vials under these conditions was completed within 2.5 hours. After removal of several vials, the temperature was increased to 50 C and the remaining samples were kept under vacuum for 7 days.

Ice nucleating activity of preserved INB was measured after the samples were rehydrated with 10 ml of 0.01 M phosphate buffer. Ice nucleating activity was measured as a concentration of ice nucleating centers that nucleate an ice crystal in a 10 ul buffer drop during 5 min at -5 C.

The ice nucleating activity of the samples that had been removed from the vacuum chamber after drying at 25 C was approximately 50% less than the initial activity of frozen-thawed INB. (The relative standard error in the measurement of ice nucleating activity is less than 20%). Because, it is known that freezing of INB does not significantly decrease ice nucleating activity, the 50% decrease of the activity observed in this experiment is probably because the additional freezing step increases sensitivity of INB to preservation by drying. At the same time, no additional decrease of the activity of the INB was observed after an additional 7 days drying at 50 C under vacuum.

(4) When stable foams containing INB, prepared as above, were subjected to milling using a modified Virtis homogenizer, there was no loss of ice nucleating activity in the rehydrated powder, compared to the rehydrated foam.

(5) A 1.5 ml tube containing a frozen (-76 C) suspension of E. coli (XL10-GOLD) from Stratagene was thawed in an ice bath. A 100 l aliquot was transferred to 50 ml of NZYM (Casein digest yeast extract medium) broth and incubated at 37 C on an orbital shaker overnight. After 14 hours of growth, 10 ml of this growth culture was inoculated into 100 ml of sterile NZYM broth to continue the culture growth at 37 C. During the culture growth the optical density (OD@620 nm) was measured every hour to determine the end of logarithmic bacteria growth. When the transition phase was reached (OD = 1 to 1.06) the cells were ready to be harvested. The culture medium (5 ml) was pipetted into a centrifuge tube and centrifuged for 10 min. The supernatant was then poured off and the weight of the pellets was measured to determine the approximate concentration of the cells.

The bacterial cells were resuspended with 5 ml of NZYM broth or preservation solution consisting of 25% sucrose and 25% fructose in MRS broth. The cells resuspended with NZYM broth were used as a control. The cells suspended in 25% sucrose and 25% fructose in MRS broth (1 ml) were placed in 20 ml glass vials and dried under vacuum similar to the INB were dried in the Example #1. After that, the samples were kept under vacuum up to 24 days at room temperature. Dried samples were assayed at selected time intervals. The survival of the preserved cells was measured after rehydration with 0.1% peptone solution in water at room temperature. To determine concentration of viable cells the suspensions were pour plated in Petri dishes at the appropriate dilution on LB Miller agar followed by incubation at 37 C for 36-48 hours. Approximately 25 ± 10% of control cells survived after drying and one day of storage under vacuum. Moreover, the portion of surviving cells did not decrease during the subsequent 24 days of storage under vacuum at room temperature.

Optional Stability-Drying – The mechanically stable foams formed during primary foam-drying, may optionally undergo secondary or "stability" drying at increased temperatures. Since Tg is dependent on the water content of the sample and since Tg increases with increased dehydration, different stability-drying protocols may be applied depending on the desired storage temperature, to generate a Tg consistent with vitrification upon cooling to that storage temperature. However, because dehydration of materials is practically impossible once they have entered the glass state, the key to

vitrification according to the present invention, where ambient storage temperatures may be desired, is to conduct the stability drying at a temperature significantly higher than the ambient temperature.

Ultimate storage temperatures are preferably within the range of 0-70 C. More preferably, common storage temperature selections are greater than or equal to 0 , 4 , 20 , 40 , and 50 C. In some cases, where refrigerated storage may be preferred, stability-drying could be carried out at room temperature followed by cooling to the storage temperature or below. In other instances, however, where stability at room temperature is desired, dehydration at a temperature above room temperature should be employed, followed by cooling to room temperature.

For any given culture to be preserved, the nature and stability characteristics of the cells will determine the maximum temperature they can withstand during the foam-drying and stability-drying steps. In some cases for example, it has been shown that after foam-drying at room temperature, the stability-drying temperature may be increased to higher temperatures without loss of viability. Selection and optimization of foam-drying and stability-drying parameters is preferably performed for each strain of bacteria to be preserved, using small sample volumes. A range of foam-drying temperatures and pressures, which are together sufficient to effect boiling of the suspension, may be tested. Once a mechanically-stable foam has formed, cell viability can be measured to determine optimal foam-drying conditions. Similarly, the temperatures and pressures to which foam-dried cultures are exposed may be varied to provide a range of stability-drying parameters. Again, cell viability serves as an indicator of optimal stability-drying conditions. In some cases, continuous or step-wise increases in the stability-drying temperature may be used to place labile cells in a state of thermal stability at storage temperatures that may be lethal for native cultures. In a preferred aspect, the stability-drying temperature is above a desired storage temperature.

In addition to conducting the stability-drying at temperatures above the selected storage temperature, it is preferred that this drying is carried out for a period of time sufficient to actually raise Tg above the storage temperature. Based on empirical results obtained with dried 10 ul drops of 15% sucrose + 15% raffinose solution, it was demonstrated that more than 12 hours of stability drying at temperatures above 70 C was required to raise Tg to above 25 C. Foam-drying in these experiments was for 12 hours at room temperature (20 C). The results suggest that extended stability-drying times (more than 12 hours at 70 C and more than 36 hours at 50 C) may be needed to effect increases in Tg over room temperature.

To ensure that the Tg is actually greater than the storage temperature, at least two methods are known for estimating Tg by thermal analysis. Differential scanning calorimetry (DSC) is the most commonly used technique. However, DSC may be unreliable for measuring Tg in samples that contain polymers. Alternatively, Thermally Stimulated Polarization Current (TSPC) methods are specifically adapted for analysis of polymers. The TSPC method is preferred because it is reliable for all samples, although it requires slightly larger sample volumes.

After foam-drying and optional stability drying, the dried foams may be stored at a selected storage temperature for a selected storage period. Following drying and/or storage, dried bacterial cultures can be rehydrated with culture medium and assayed for viability. Typically, viable cell counts are determined by serial dilution of the sample and plating on appropriate agar. The plates are incubated and colony-forming units (CFU) determined. Percent

survival can be calculated by dividing the surviving CFU/ml of the sample being tested by the CFU/ml of the original fermentation.

Modification of Fermentation to Enhance Desiccation Tolerance – The fermentation process can be defined as a chemical transformation of organic compounds by the metabolic activity of the microorganisms. Numerous commercially important products are made by fermentation, including microbial cells (starter cultures), large macromolecules (enzymes, gums, etc.), primary metabolic end products (lactic acid, flavor compounds, etc.), and secondary metabolites (antibiotics, etc.).

There are two major kinds of fermentation processes, traditional and controlled. Traditional fermentation is driven by the metabolic activities of the natural flora associated with the raw materials. In controlled fermentation, the desired microorganisms, so called "starter cultures", are added to the raw materials and then the appropriate environmental conditions are set. Industrial fermentation is exclusively operated as controlled processes, where the use of defined starter cultures increases the probability for success of the process and ensures high consistency of the product.

For the purposes of the present disclosure, microbial fermentation is considered a multi-dimensional process, comprising many parameters including; culture temperature, pH, osmotic strength, time of cell growth (cell density), ionic strength, concentration of bivalent cations, and media composition (e.g., nutrient, oxygen, nitrogen concentration, etc.). Optimal fermentation conditions are those which result in maximal bacterial growth, metabolic activity and cell density (fermentation yield). However, these optimal fermentation conditions usually do not result in a culture containing cells that exhibit optimal desiccation tolerance. Our approach is to find the combination of fermentation parameters that would yield maximal cell survival after fermentation, preservation by foam-drying (with or without optional stability-drying), and subsequent storage at temperatures required for the practical application of the particular cell strain.

Bacteria have developed numerous mechanisms to cope with non-optimal growth environments. The exposure of cells to different stresses is known to have an immediate impact on bacterial physiology. We have found that the growth of cellular (bacterial) cultures under certain sub-optimal fermentation conditions increases the desiccation tolerance of many bacterial strains. In contrast the previous work of Legg (U.S. Pat. No. 5,728,574), a more effective means of enhancing desiccation tolerance may be found by the simultaneous modification of all or several different fermentation parameters.

Temperature – Most bacterial species are able to grow over a wide range of temperatures, up to 40°C. The effect of temperature on bacterial growth has kinetics similar to that on the rate of a chemical reaction and which is described by the so called "Arrhenius plot" (curve has hyperbolic shape). The optimal, maximal, and minimal growth temperatures are called "cardinal temperatures". The optimal temperature is defined as the temperature at which bacterial growth occurs at a maximal rate. The growth temperature range between the maximum and optimal temperature is called the "high growth range". The growth range between the minimum and the optimal growth temperature is called the "low growth range". Bacterial growth is linear in the optimal temperature range. The slope

of the growth curve increases in the low and high growth range, and becomes vertical at inhibitory (lethal) temperatures.

The nutritive characteristics of the growth medium can affect growth at the low and high temperature ranges, but have no effect on the optimal temperature of growth. Enriching the growth medium is known to have an effect on the maximum growth temperature of E. coli, but not on the minimum growth temperature. Based on temperature ranges where growth occurs, bacteria are classified as "psychrophiles" (can grow at +5° C or below, and up to 40° C), "mesophiles" (growth range: from +5° C to 45-50° C, optimal growth at 37° C; examples E. coli, Lactobacilli, etc.), and "thermophiles" (growth range from 40° C to above 100° C).

The growth rate decreases rapidly at temperatures above or below the optimal. In addition, there are precise temperatures at which bacterial growth does not occur. When exposed to increased temperatures, bacteria activate their "heat shock" stress responses, change composition of the phospholipids in biological membranes, etc. The maximum growth temperature of many bacteria is determined by the thermal instability of their proteins. In contrast, minimum growth temperature is set by factors that cause weakening of hydrophobic bonds involved in higher levels of protein structural organization.

pH – Based on their response to external pH, bacteria are classified as "acidophile" (grow best at low pH), "neutrophile" (grow best at neutral pH, like E. coli), and "alkalophile" (grow best at pH above 7.0). Bacteria can grow over a wide ranges of pH's. However, bacteria maintain their internal pH near a fixed optimal rate, which is defined by the optimal pH for catalytic activity of the enzymes necessary for bacterial growth. Depending of the pH of the growth medium, the bacterial cytoplasm could be either more acidic or more alkaline than the medium. For example. E. coli can grow over pH ranges of 6.0 to 8.0, but always maintains an internal pH at 7.6. The Lactobacillus species are known to tolerate external pH as low as 3.5, but these bacteria maintain the internal pH of 7.6. However, because they have extremely efficient proton pumps, Lactobacilli can survive internal pH as low as 4.4. Efficient pumping-out of protons from the cytoplasm seems to be the general strategy that bacteria developed to cope with a low pH.

Osmotic pressure – All bacteria, with the exception of mycoplasmas, have developed versatile strategies to maintain a characteristic turgor pressure over a relatively broad range of osmotic strengths of their external environment. Generally, Gram-positive bacteria maintain higher turgor (5-22 atm) than Gram-negative bacteria (0.8-5 atm). Turgor pressure is maintained mostly by adjusting the intracellular concentration of so called "compatible solutes", small, neutral organic molecules, which are highly soluble and do not alter cytoplasmic functions. Compatible solutes can be accumulated by either de novo synthesis or by transport into the cells after osmotic shock. Many different compounds can function as compatible solutes, including betaine (N, N, N-trimethylglycine), carnitine, trehalose, sucrose, glucitol, ectoine, mannitol, proline, glycerol, small peptides, etc. (reviewed by Csonka. 1991 Annu. Rev. Microbiol. 45:569-606). Some of the listed compounds are also widely known as "fillers" (i.e., trehalose, sucrose, manitol, glycerol, and glucitol).

Cell Density ("pheromones" or "autoinducers") – Many bacteria have developed mechanisms for sensing and responding to an increase in population density (Gary M. Danny & Stephen C. Winans, Eds. 1999 Cell-Cell Signaling in

Bacteria, ASM Press, Washington, DC). High cell density, so called "quorum sensing", is known to contribute to the regulation of many important physiological processes. The development of genetic competence, sporulation, bioluminescence, conjugation, plant and animal pathogenesis, production of bacteriocines and antibiotics, are some of the processes regulated by cell-cell communication which occurs when the bacterial population reaches high density. Many different signaling molecules have been described, but it is generally accepted that homoserine lactones (HSLs) mediate cell-cell communication in Gram-negative bacteria and that different small peptides mediate cell-cell communication in Gram-positive bacteria. Signaling molecules, so called "pheromones" or "autoinducers", are produced and secreted into the growth medium at a basal level at low cell densities. The concentration of the pheromones increases with cell density until a threshold level is reached, and could be recovered from the supernatant after centrifugation of the culture. Pheromones usually enter the cells via diffusion or by a dedicated transport system. Within the cell, signaling molecules interact with different effector molecules directly or via a two-component sensing systems consisted of His-Kinase and a response regulator protein. Cell-cell communication was described in several bacterial genera, including Vibrio, Streptococcus, Enterococcus, Pseudomonas, Myxococcus, Bacillus, Agrobacterium, Erwinia, Rhizobium, Xanthomonas, Staphylococcus, Lactococcus, Lactobacillus, and Streptomyces.

Divalent Cations – The composition of the fermentation broth will include a large number of divalent cations. These cations are involved in a wide range of functions, ranging from cofactors in enzymatic reactions to providing covalent bonding sites in cell wall formation. For example, the addition of 0.1% Ca^{++} to L. acidophilus ATCC 4356 changes the colonial morphology from a mixture of "rough" and "smooth" colonial forms to almost completely "smooth" forms. "Smooth" colonies are composed of small bacilloid cells while "rough" colonies are composed of filamentous cells. It has been shown that the "smooth" form survives freeze-drying at higher yields than the "rough" form. Wright and Klaenhammer 1981 Calcium-Induced Alteration of Cellular Morphology Affecting the Resistance of Lactobacillus acidophilus to Freezing. Appl. Environ. Microbiol. 41:807-815.

The enhancement of cell desiccation tolerance by modifications of fermentation conditions may be more fully appreciated with reference to the following working examples:

Fermentations were performed using a New Brunswick Scientific Company BioFlo 2000 fermenter with a 2 L working capacity. The fermenter was equipped with a pH module to control pH by the addition of acid or base to the fermenting culture as necessary.

Preservation was carried out generally as described above by foam-drying. More specifically, the starting shelf temperature was set to 7° C. Bacterial cells were suspended in a concentrated sucrose solution and aliquoted into serum vials. A thermocouple was placed into one of the samples to monitor sample temperature during the preservation process. The vacuum chamber was closed and the pressure reduced to 5 Torr. After sample temperature had dropped to about 0° C, due to evaporational cooling, the shelf temperature was increased to 20° C. The pressure was dropped incrementally such that the sample temperature never fell below –10° C. The final pressure was 200 mTorr. After a mechanically-stable foam had formed, the shelf temperature was increased to 25° C for stability-drying. After 15 hours at 25° C, the shelf temperature was increased again to 45° C. The samples

remained under vacuum at 45° C for 36 hours. The samples were sealed under vacuum prior to being removed from the vacuum chamber.

After storage for the indicated time periods under the indicated conditions, cells were re-hydrated with buffer and assayed for viability. Viable cell counts were determined by serial dilution of the sample and plating on appropriate agar. The plates were incubated and colony-forming units (CFU) determined. Percent survival was calculated by dividing the CFU/mL of the sample being tested versus the CFU/mL of the original fermentation.

Example 1

Fermentation of Lactobacillus acidophilus (ATCC 4356) at Low pH

Lactobacillus acidophilus ATCC 4356 (L. acidophilus) is a commercially significant species. L. acidophilus grows by fermentation of lactose, glucose and a range of carbohydrates. The end product of this fermentation is almost exclusively lactic acid. If the lactic acid produced is not neutralized by the addition of base, the pH of the culture decreases. L. acidophilus, and other lactic acid bacteria will produce acid to the point their growth is curtailed by the low pH.

Conventional fermentation calls for growth on complex media (Difco MRS broth + 0.05% cysteine) under pH-regulation between 5.80-6.00 at 37° C. The fermentation is anaerobic. The broth was inoculated with cells from a frozen seed. The cells were fermented to stationary phase indicated by an O.D. of 2.4. Preservation survival following foam-drying and rehydration was less than 20%. Bacteria were enumerated by plating on MRS+0.05% cysteine agar and incubating for 48 h at 37° C under anaerobic conditions.

L. acidophilus ATCC 4356 was also fermented in a modified manner by allowing the pH of the culture to fall with no regulation. The cells were fermented to stationary phase indicated by an O.D. of 2.4. The conditions were identical to the conventional fermentation but the pH was allowed to drop to the point it restricted growth. The survival of these cells (no pH regulation) following foam-drying and rehydration was over 70%.

L. acidophilus fermented with no pH regulation to an O.D. (@600 nm) greater than 2.4 and a final pH of lower than 4.0 was dried with a 70% level of survival after re-hydration.

Intermediate levels of survival after foam-drying and rehydration, between 20% and 70%, were observed when L. acidophilus was grown under conditions in which the cultures did not completely achieve and O.D. of greater than 2.4 and a pH lower than 4.0. If the O.D. exceeded 2.4, then the pH was lower than 4.0, and vice-versa. The enhanced desiccation tolerance was observed when the samples were foam-dried in both 20 uL drops and in foams of larger volumes.

Example 2

Effect of Growth Phase on Survival Yields of L. acidophilus

Cultures were started from a frozen seed. The cells were fermented in MRS+0.05% cysteine broth at 37° C. Bacteria were harvested in logarithmic phase of growth (defined by the mid point in exponential increase in O.D. (optical density by absorbance at 600 nm) and in stationary phase (defined by no increase in O.D.). The bacteria were

preserved by foam-drying. Bacteria were enumerated by plating on MRS+0.05% cysteine agar and incubating for 48 h at 37° C under anaerobic conditions.

The results illustrated in Figure 1, show that preservation yields for both log and stationary phase cells was 40%. The first two time points (prior to the zero time point) show cell counts prior to foam-drying and following the drying process, respectively. Clearly, after drying, the cell counts remained essentially stable throughout the 17 day storage period at 37° C. The cell density was $4.2x10^8$ CFU/mL in the log phase versus $1.0x10^9$ CFU/mL in the stationary phase.

Example 3

Effect of Ca^{+2} on Desiccation Tolerance of L. acidophilus

Bacteria were fermented in Difco MRS+0.05% cysteine broth with the addition of 0.1% Ca^{+2} at a temperature of 42° C. The pH was maintained at 5.80. Cells were harvested in stationary phase as determined by a stabilization of optical density. The bacteria were preserved by foam-drying. Bacteria were enumerated by plating on MRS+0.05% cysteine agar and incubating for 48 h at 37° C under anaerobic conditions.

Preservation survival was 47-50%. Untreated cells had a survival if less than 40%. Bacteria fermented under the previously described conditions at 37° C with added Ca^{+2} alone had preservation yields of about 50% and were stable for 16 days at 50° C.

Example 4

Effect of Depleted Supernatant on Desiccation Tolerance of L. acidophilus

Depleted supernatant was prepared by inoculating a 200 mL stir flask containing MRS+0.05% cysteine with frozen seed. This culture was incubated at 37° C with no pH regulation until the optical density (at 600 nm) was greater than 2.60 and the pH of the culture was less than 4.0. The culture was removed from the stir and centrifuged. The supernatant was decanted off the pellet. The collected supernatant was filtered through a 0.22 um filter. Samples of the depleted supernatant were neutralized by the addition of 1 M NaOH until the pH reached 7.0.

Cells were fermented in MRS+0.05% cysteine broth under pH regulation (pH=5.80) at 37° C. Logarithmic phase cells were treated with depleted supernatant by centrifuging the samples and re-suspending the cells in a depleted supernatant from a previous unregulated fermentation. The bacteria were preserved by foam-drying. Bacteria were enumerated by plating on MRS+0.05% cysteine agar and incubating for 48 h at 37° C under anaerobic conditions.

The treatment with depleted supernatant increased the preservation survival of log phase cells from 5-10% to 20-30%. The observed increase in preservation survival after treatment with depleted supernatant was less in stationary phase cells compared to log phase cells fermented under pH regulation. The effect of the depleted supernatant was observed with neutralized as well as native depleted supernatant

Example 5

Desiccation Tolerance of L. acidophilus DSM strain

Bacteria were fermented in Difco MRS+0.05% cysteine broth with the addition of 0.1% Ca^{+2} at a temperature of 42° C. The pH was regulated at 5.80. Cells were harvested in stationary phase as determined by a stabilization of optical density. The bacteria were preserved by foam-drying with a yield of 55%. Bacteria were enumerated by plating on MRS+0.05% cysteine agar and incubating for 48 h at 37° C under anaerobic conditions.

The preserved bacteria were prepared as described. The mechanically-stable foam glass was milled to a fine powder. The milled glass containing the preserved bacteria was mixed with an inulin powder under dry conditions (r.h. < 20%). The mixture was sealed in foil pouches and stored at room temperature. The bacteria were preserved with a yield of 62%. As illustrated in Figure 2, the bacteria in the mixed powder were stable for 86 days at 25° C.

Example 6

Growth to Stationary Phase of Salmonella choleraesuis

S. choleraesuis was fermented by conventional methods in M-broth comprising Difco M-broth (36 g), Tris 7-9 buffer salt (12.0 g), 10% Phenol red (2.0 mL), deionized water to 1000 mL and adjusted to pH 7.4. Fermentation pH was adjusted to 7.1 by the addition of 1N HCl or 1N NaOH. Stationary phase was defined as the stabilization of optical density at 650 nm. The cells were harvested and concentrated by centrifugation before preservation. The bacteria were preserved by foam-drying. Bacteria were enumerated by plating on Difco Trypticase Soy Agar (TSA) and incubating for 24 hours at 37° C.

Preservation survival after drying was 10-30% when the cells were harvested in late logarithmic phase or early stationary phase (defined as the first 60 minutes of stationary phase). Preservation survival after drying was highest three hours in to stationary phase. Preserved bacteria were stable for 200 days stored at 4° C.

Example 7

Growth to Stationary Phase of Bordetella bronchiseptica

B. bronchiseptica cultures were fermented by on Dextrose Starch broth at 37° C under aerobic conditions. Dextrose Starch Broth is composed of dextrose (2.0 g), soluble starch (10.0 g), NaCl (5.0 g), disodium phosphate (3.0 g), gelatin (bacteriological) (20.0 g), glycerol (10.0 g), sodium acetate (0.08 g), deionized water to 1000 mL and pH adjusted to 7.3. The culture was grown to late stationary phase as defined by the stabilization of O.D. The bacteria were preserved by foam-drying. The bacteria were enumerated by plating on Bordet Gengou + 5% blood agar and incubating at 37° C under aerobic conditions. Survival after drying was greater than 90%. Viability of the dried samples was stable for over 70 days at 37° C. Parallel cultures were dried by conventional freeze-drying. The results illustrated in Figure 3 show that cells preserved by freeze-drying rapidly loose their viability upon storage at 37° C in comparison to cell preserved by foam-drying.

Example 8

Fermentation to Stationary Phase of Lactococcus lactis cremoris DSM Strain

Bacteria were fermented in a skim milk-based medium at 30° C. The fermentation pH was maintained at 5.80 by the addition of 4.76% NH_4OH. The fermentation was harvested at stationary phase as defined by 90 minutes after the last addition of base to the culture. The bacteria were preserved by foam-drying. The bacteria were enumerated by plating on TSA and incubating at 30° C for 48 hours. The cells were preserved with a yield of 100%. As illustrated in Figure 4, the preserved bacteria were stable for 108 days at 25° C and 29 days at 37° C.

Example 9

Effect of Osmotic Pressure on Desiccation Tolerance of

Lactococcus lactis subsp. cremoris ATCC 19257

Lactococcus lactis subsp. cremoris ATCC 19257 was fermented in skim milk-based medium at 30° C. The pH of the culture was maintained at 5.80 by the addition of 4.76% NH_4OH. The cells were harvested in stationary phase as defined by the halt in addition of base to the culture. The bacteria were preserved by foam-drying. The bacteria were enumerated by plating on TSA and incubating at 30° C for 48 hours. After drying the cells fermented under these conditions, survival was less than 18%.

The conventional fermentation described above was modified by the addition of a non-metabolized sugar (sucrose) to the fermentation broth. Sucrose was added at a concentration of 20%. Lactococcus lactis subsp. cremoris ATCC 19257 was fermented as above in a broth of MRS+C broth +20% Sucrose. The preservation survival after drying was 100%.

WHAT IS CLAIMED IS:

1. A scaleable method for preserving bacterial cells in a dried state, comprising:

culturing the bacterial cells under modified fermentation conditions that inhibit fermentation yield relative to optimal fermentation conditions and also enhance the cells' ability to survive dehydration; and

drying the cell suspension by boiling under vacuum to form a mechanically-stable foam.

2. The method of Claim 1, wherein the modified fermentation conditions comprise fermentation parameters selected from the group consisting of temperature, pH, osmotic pressure, divalent cation concentration, cell density, nutrient concentration, oxygen concentration, and nitrogen concentration.

3. The method of Claim 2, wherein two or more of the fermentation parameters are modified.

4. The method of Claim 1, wherein the modified fermentation conditions are applied for only a portion of the culturing step.

5. The method of Claim 1, wherein the modified fermentation conditions comprise at least increasing osmotic pressure of the media to 1.2-10 times isotonic pressure.

6. The method of Claim 5, wherein the osmotic pressure is increased by adding non-permeable solutes.

7. The method of Claim 5, wherein the osmotic pressure is increased by adding permeable solutes.

8. The method of Claim 5, wherein the osmotic pressure is increased by adding metabolized solutes.

9. The method of Claim 5, wherein the osmotic pressure is increased by adding at least one product of cell metabolism.

10. The method of Claim 1, wherein the modified fermentation conditions comprise a decrease of about 0.5-4.5 pH units pH from an optimal pH.

11. The method of Claim 1, wherein the modified fermentation conditions comprise maintaining cultures to late stationary phase.

12. The method of Claim 1, wherein the modified fermentation conditions comprise maintaining cultures to late logarithmic phase.

13. The method of Claim 1, wherein the modified fermentation conditions comprise a change in temperature within a range of 1-15° C from optimal.

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(54) Title: PRESERVATION OF BACTERIAL CELLS AT AMBIENT TEMPERATURES

(57) Abstract: The present invention is related to enhancing the survival of bacterial cells during drying and storage by modifying their culture conditions prior to foam-drying. The modified culture conditions may result in the generation of protectants.

Figure 1:Stability of Preserved *L. acidophilus* ATCC4356
Cells Harvested in Log or Stationary Phase

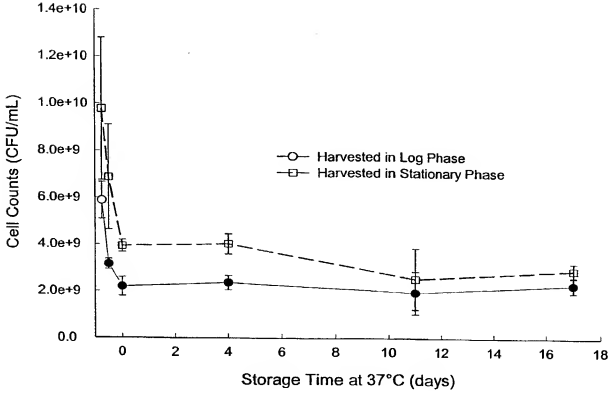


Figure 2:Stability of Preserved DSM *Lactobacillus acidophilus* Mixed with Prebiotic and Stored in Sealed Pouches at Room Temperture

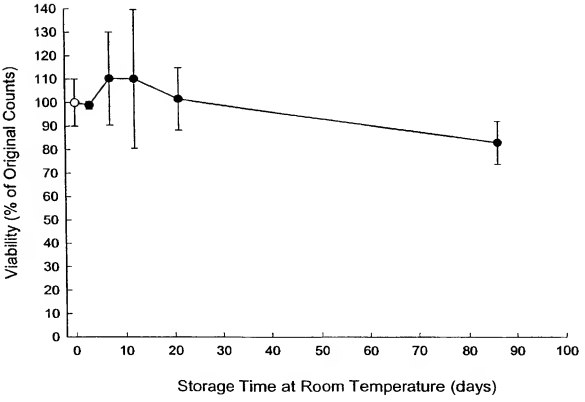


Figure 3: Stability of *Bordetella* after Preservation Using the VitriLifeSM Process and by Commercial Freeze-Drying Stored at 37°C

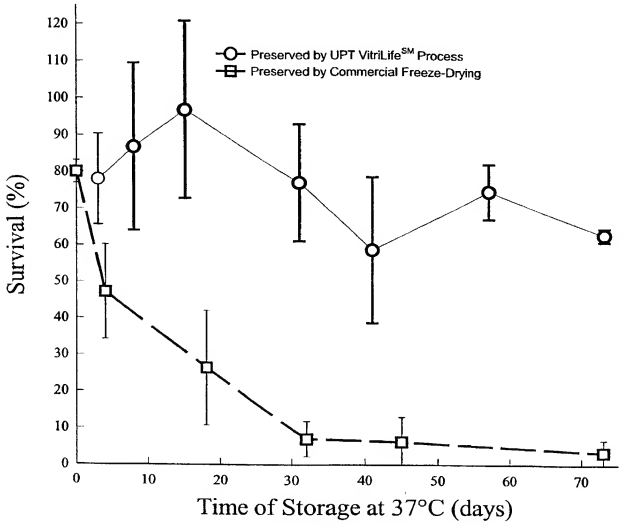
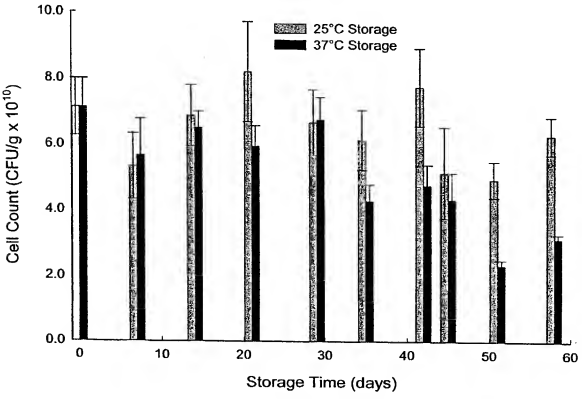


Figure 4: Stability of Preserved DSM *Lactococcus lactis sbsp. cremoris*
Samples had been stored for 50 days at 25°C before study



#17

Fax to Mark Benedict -

Page 1 949 760 9502 Attorney's Docket No. UPTINC.021A

DECLARATION - USA PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled PRESERVATION OF BACTERIAL CELLS AT AMBIENT TEMPERATURES; the specification of which was filed on **March 22, 2002** as Application Serial No. **10/089,003.**

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56;

I hereby claim the benefit under Title 35, United States Codes § 119(e) of any United States provisional application listed below.

Application No.: 60/149,795 Filing Date: August 19, 1999

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)			Priority Claimed
No.: **PCT/US00/40704**	Country: **PCT**	Date Filed: **August 21, 2000**	**yes**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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